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PRINCIPAL INVESTIGATOR: Yong-Mei Zhang, PhD

CONTRACTING ORGANIZATION: Medical University of South Carolina
Charleston, SC 29425

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Yong-Mei Zhang, Xiangdong Bi, Kristopher Virga, Wei Chen, Jordon Gruber, Bo Wang

E-Mail: zhangym@musc.edu

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14. ABSTRACT

During this performance period, we have made significant progress on the following Tasks as stated in SOW: 1, 2, 4, 5, 6, 7 and 8. For Specific Aim 1, we have synthesized 18 QSI-PAMAM complexes by encapsulation (Task 1), conjugation (Task 2), or both. For Specific Aim 2, we have established all the protocols to measure planktonic and biofilm growth of PA (Task 5), turnover of PAMAM in PA (Task 6), quinolone signals by HPLC-MS (Task 7), and extracellular virulence factors (Task 8). The most significant finding was the specific inhibition of PAMAM dendrimers on *Pseudomonas aeruginosa* (PA) biofilm formation and growth. This is significant because PA in the biofilm form is known to be up to 1000 fold more resistant antibiotic treatments. We systematically analyzed the effects of surface charge and particle size on PA biofilm. PAMAM dendrimers with more positive charges on the surface and bigger particle sizes exhibited more potent inhibition. Confocal fluorescent microscopy showed that PAMAM dendrimers also altered the morphology of PA biofilm. In addition, combination of PAMAM dendrimers also significantly enhanced the inhibition of PA biofilm growth by antibiotic such as amikacin.

15. SUBJECT TERMS
Pseudomonas aeruginosa (PA), Biofilms, Quorum-sensing inhibitors, PAMAM dendrimers, Nanoparticles.

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INTRODUCTION:

The objective of this research is to develop topical treatment of *Pseudomonas aeruginosa* (PA) wound infections using quorum-sensing inhibitors complexed with poly(amidoamine) (PAMAM) dendrimers. We hypothesize that, by combining the novel treatment approach (QS inhibitors, QSI) with a superior topical drug delivery vehicle (PAMAM dendrimer conjugates), we can overcome the limitations associated with current topical antibacterial agents. The proposed studies are organized in the following three specific aims:

1. To synthesize PAMAM dendrimer complexes with QS inhibitors (QSI-PAMAM).
2. To determine the effects of QSI-PAMAM on PA virulence *in vitro*.
3. To determine the antibacterial effects of QSI-PAMAM complexes in PA infection models.

BODY:

Research accomplishments during this perform period (Year 1) are organized according the approved Tasks in the SOW (underlined). Tasks for both Specific Aims 1 and 2 were planned for Year 1. Tasks for Specific Aim 3 were planned for Year 3, therefore the Aim 3 is not included in this report.

Specific Aim 1. To synthesize PAMAM dendrimer complexes with QS inhibitors (QSI-PAMAM)

Task 1. Formation of QSI-PAMAM by encapsulation
Task 2. Formation of QSI-PAMAM by covalent attachment (conjugation)

We purchased anthranilic acid and homoserine lactone analogs (3CABA, 4CABA, 6CABA, 6FABA, SA, nifuroxazide, baicalein, baicalin) and PAMAM dendrimers (G5-NH_2, G4-NH_2, G3-NH_2, G5-COOH, G5-OH) from commercial sources.

To synthesize QSI-PAMAM complexes by encapsulation, PAMAM dendrimers were dissolved in methanol, to which a methanol solution of QS inhibitor was added (**Figure 1a**). The mixture was allowed to stir overnight for encapsulation to complete. The resulting encapsulated dendrimer product was obtained by membrane filtration through a 10 kD molecular weight cutoff (MWCO) membrane to remove the free small molecules. After washing sequentially by methanol, PBS buffer and water, the solid product of the encapsulated QSI-PAMAM was obtained by lyophilization. Characterization of encapsulated dendrimer species was carried out by HPLC and ^{1}H NMR as described previously (1). The number of inhibitors encapsulated in each dendrimer molecule (ratio of QSI/PAMAM) was determined by proton integration of the small molecule and the dendrimer using NMR. **Figure 1b** shows a representative NMR spectrum of 6CABA complexed with G5-NH_2 (B3-17). Successful entrapment of 6CABA in the dendrimer was demonstrated by the changes of the chemical shifts of 6CABA protons in the complex (6.98, 6.75 and 6.73 ppm) from those in the free form (7.22 and 7.08 ppm, **Figure 1b** inset), indicating the hydrogen bond formation between the carboxylic acid (and amino group) of 6CABA with the dendrimer. Based on proton integration of the ^{1}H NMR, we determined that 65 molecules of 6CABA are encapsulated in each dendrimer molecule in this complex. The high molar ratio values suggest that the use of QSI-PAMAM will significantly increase the payload of the inhibitors. The NMR chemical shift calculation was performed for the other synthesized QSI-PAMAM complexes by encapsulation, and the ratio of QSI and PAMAM in the complex was listed in **Table 1**.

Conjugation of baicalin was carried according the proposed synthesis scheme. In this performance period, we synthesized baicalin complex with G5-Ac50 by conjugation. To generate covalently conjugated QSI-PAMAM complexes, the PAMAM dendrimer G5 was partially acetylated to reduce surface positive charge from the amine groups, thus reducing toxicity of the dendrimer and non-specific binding to eukaryotic cells. Baicalin bears an active hydroxyl for dendrimer conjugation, baicalin-PAMAM complex was synthesized in three steps based on a reported procedure (2). We made two batches of baicalin-G5Ac50 complex by conjugation, B3-24 and B4-5. Drug load was significantly improved in the second try. To further increase the drug load in the complex, we encapsulated more baicalin molecules into complex B4-5 to yield B4-6, which contains 40 baicalin molecules per molecule of dendrimer.

Table 1 lists all the QSI-PAMAM complexes we synthesized in this performance period. In summary, we synthesized 18 QSI-PAMAM complexes by either encapsulation, conjugation, or both.

Task 3. Large scale synthesis of QSI-PAMAM for in vivo studies

Because large-scale synthesis is not required for the in vitro analyses for Specific Aim 2. We did not produce QSI-PAMAM in large quantity in Year 1.

Specific Aim 2. To determine the effects of QSI-PAMAM on PA virulence in vitro.

Task 4. To Determine the effects of QSI-PAMAM on PqsA activity

PqsA is the first enzyme in quinolone synthetic pathway (3). Anthranilic acid (AA) is a substrate for PqsA. AA analogs, such as 4CABA, 6CABA and 6FABA, inhibit PqsA activity by competing with its cognate substrate (4). The effect of QSI-PAMAM on PqsA activity was carried by an assay as described (5). Our results showed that complexation of AA with dendrimers did not significantly alter the inhibitory effect of AA analogs on PqsA. This result was expected because the advantage of dendrimer as a drug carrier is to increase compound solubility/uptake by the cells and the PqsA enzyme assay was done in vitro with purified recombinant PqsA protein. On the other hand, the AA analogs are poorly soluble in water, but upon encapsulation with PAMAM dendrimer nanoparticles, the QSI-PAMAM complex was freely soluble in water, demonstrating significantly enhanced solubility. Therefore, we expect to see significant improvement of AA analog activity in cell-based assays.

Task 5. Effects of QSI-PAMAM on PA growth

Although the quorum-sensing (QS) network is essential for PA pathogenesis, it is not required for planktonic bacterial growth. However, because QS promotes biofilm formation, QSIs could reduce biofilm formation, which occurs in the early stage of PA infections in wounds (6). Biofilms are made of a matrix of extracellular polymeric substances (DNA, proteins, polysaccharides) that form a shield around the bacteria aggregates, which are even more resistant to antibiotics than free-living cells (7). Therefore, we tested the effect of QSI-PAMAM on PA growth in both planktonic and biofilm forms. Planktonic growth of PA was monitored by measuring the optical density of the culture suspension at 600 nm. We established a novel method to monitor PA biofilm growth using 96-well microtiter plates with pegged lids (8). The workflow of this method is shown in **Figure 2**. The pegged lid method allows bacterial biofilm to attach to the pins, and the 96-well format allows us to test different QSI-PAMAM complexes at different concentrations simultaneously.

Figure 3 shows results of 4 QSIs and their complexes with PAMAM on PA biofilm growth.

No inhibition on PA planktonic growth was observed. Among the 4 QSIs tested (6CABA, 4CABA, 3CABA, SA), 6CABA-G5Ac50 (B3-17) showed significantly more potent inhibition on PA biofilm in comparison to 6CABA alone.

While performing the dendrimer only controls in these experiments, we observed some inhibition of PA biofilm by the particles, especially those with more positive charge on the surface. G5-NH_2 dendrimers contain over 100 surface amine groups, which are positively charged. We performed experiments to systematically investigate the effects of surface charge and particle size on PA biofilms. There results were summarized in **Figures 4-6**. Due to the different chemical properties of the end groups, the surface charge of various PAMAM particles is different. G5-NH_2 particles are positively charged, G5-OH neutral, and G5-COOH particles are negatively charged. Previous toxicity studies in mammalian cells showed that PAMAM particles with positive charges can lead to cell death (9). Therefore, to ensure that the charge of the particles does not cause toxicity in PA, we tested the effects of different PAMAM dendrimers, including G5-NH_2, G4-NH_2, G5-OH, G5-NH_2(Ac100), and G5-NH_2(Ac100)-PEG, on PA planktonic and biofilm growth. Of the five different dendrimers tested at 500 μg/ml, no inhibition was observed on PA planktonic growth. Interestingly, the positively charged G5-NH_2 and G4-NH_2 unmodified dendrimers decreased biofilm formation significantly, whereas the 3 neutral particles, G5-OH, G5-NH_2(Ac100), and G5-NH_2(Ac100)-PEG, did not inhibit PA biofilm formation (**Figure 4**). Negatively charged G5-COOH was also tested and did not exhibit inhibition on both PA planktonic growth and biofilm formation (**Figure 4b**). These results suggested that positively charged PAMAM dendrimers would provide added benefits as a delivery vehicle of QSI compounds to inhibit PA biofilms, by both increasing the transport of QSI as drug carriers and decreasing biofilm attachment themselves. Moreover, these results showed that the inhibitory effect of the positively charged particles was more profound with the bigger G5-NH_2 (**Figure 4b**), suggesting that PAMAM dendrimers with larger diameters are more potent inhibitors of PA biofilm formation.

To further test the effects of the positive charge on PAMAM particle surface, we modified the end amine groups of G5-NH_2 by acetylation to neutralize 25% (G5-Ac25), 50% (G5-Ac50), 75% (G5-Ac75), and 100% (G5-Ac100) of the surface charge. These particles, along with un-modified G5-NH_2, were tested for inhibition against PA biofilm formation. The results showed that particles with less positive charge exhibited reduced inhibition on PA biofilm formation (**Figure 4a**). The inhibition on PA biofilm growth could be the result of interaction with negatively charged extracellular materials in the biofilm matrix, such as proteins and DNA. On the other hand, G5-NH_2 with more 50% of the amine groups acetylated did not inhibit PA planktonic growth, suggesting that the positive charged amine groups on the G5-NH_2 is inhibitory on PA planktonic growth by the positively changed dendrimers possibly by interacting with the negatively charged membrane surface.

We also used confocal fluorescent microscopy to determine the effects of PAMAM dendrimers on PA biofilm morphology. Under low shear conditions, PA forms mushroom-shaped biofilms (10). We established a protocol to monitor PA biofilm morphology on glass slides. Briefly, overnight culture of PAO1-pSMC2 expressing green fluorescent protein GFP (11) was grown in LB supplemented with 20 μM dendrimers or solvent control. A sterile glass slide was placed in the culture tube with the bottom half of the slide in the liquid culture to allow PA biofilm to form at the air liquid interface. After incubation at 37°C for 18h, the glass slide was carefully removed and placed in a sterile petri dish. PA biofilm was analyzed using Olympus Fluoview FV10 laser scanning confocal microscope. **Figure 5** shows that untreated control PA formed biofilms with

patches of high GFP density. G5-Ac50-treated (20 μM) showed a more uniform pattern; while G5-NH_2 treated samples exhibited a unique punctuate profile. These results showed that PAMAM dendrimers can alter the morphology of PA biofilm.

Because PA biofilms exhibit higher resistance to antibiotics, we tested the effect of combining aminoglycoside antibiotic, amikacin, with PAMAM dendrimers on PA growth. Results showed that addition of 10 μM G5-Ac50 (no inhibition on PA planktonic and biofilm growth) enhanced the inhibition of amikacin on PA (**Figure 6**). The enhancement was more profound on PA biofilm, tested using both pegged lid method and well biofilm method (12). This result is significant because it shows that PAMAM can also be used to improve the activity of current antibiotics, which are ineffective in killing PA biofilms.

Task 6. Turnover of QSI-PAMAM in PA

We determined the uptake and turnover of PAMAM in PA biofilms. Briefly, PA was grown statically in 96-well plates to form biofilm on the bottom of the plate. FITC-labeled dendrimer (FITC-G5-Ac75) was added to the growth medium at 20 μM and cells were incubated for 2 hr in the presence of FITC-G5-Ac75. The biofilm was washed in PBS and incubated in PBS for another 4 hr. Aliqutes of the PBS solution was removed to monitor the release of PAMAM from PA biofilm. Our results showed that about 8% of particles were internalized by PA biofilm after 2-hr incubation (**Figure 7a**). The most release of the dendrimers were observed within first 3 hr incubation in PBS (**Figure 7b**).

Task 7. Measurement of secreted QS signals

All AA analogs significantly inhibited secreted quinolone signals but not the acylhomoserine lactone signals using HPLC-MS.

Task 8. Characterization of selected QS-dependent virulent factors

We have established a quantification method for extracellular virulence factors such pyocyanin (13). The effects of QSIs on pyocyanin production was determined and results were shown in **Figure 8**. As expected, none of the QSIs (tested at 1 mM) exhibited inhibition of PA planktonic growth. Among AA analogs (Figure 8a), 6FABA and 4CABA showed the strongest inhibition on PA pyocyanin production. On the other hand, HSL analogs exhibited weaker inhibition, with baicalin as the best inhibitor (50% inhibition). We are in the process to determine the effect of QSI-PAMAM complexes on virulence factor production in comparison to QSI alone and PAMAM alone controls.

In reviewing recent literature, we identified a group of cinnamaldehyde compounds and their analog *p*-coumaric acid, which have been suggested by other research group to have putative antagonistic effects against PA quorum-sensing. We purchased 5 cinnamaldehyde compounds and *p*-coumaric acid from a commercial source and determined their effects on PA growth and pyocyanin production. Our results showed no significant inhibition of these compounds against PA planktonic growth, biofilm formation, and pyocyanin production at both 25 μM and 100 μM. These compounds were previously tested in *Vibrio harveyi* and their inhibitory effect on PA quorum sensing was implied based on the sequence homology

between the Vibrio and PA quorum-sensing regulators. The lack of activity in PA could again be the result of inability to cross the PA cell envelope.

KEY RESEARCH ACCOMPLISHMENTS:

- Synthesis of 18 QSI-PAMAM complex and analytical characterization by NMR.
- Established all the necessary methods for in vitro assays of QSI-PAMAM activities.
- Characterization of PAMAM inhibitory effects on PA biofilms.
- Quantification of PAMAM turnover in PA cells.

REPORTABLE OUTCOMES:

- We used the preliminary results from the method on the HPLC-MS analysis of quinolone to successfully apply for a feasibility pilot award from the Cystic Fibrosis Foundation. See below for details of this award. The goal of this research is to determine the role of quinolone DHQ in PA chronic infections in the lungs of cystic fibrosis patients.

  Cystic Fibrosis Foundation Pilot and Feasibility Award (ZHANG12I0)
  "The role of DHQ in chronic *Pseudomonas* infection in cystic fibrosis"
  Role: PI
  04/01/2012 – 3/31/2013 (Total cost: $43,200)

CONCLUSION:

In summary, during this performance period, we have made significant progress on the following Tasks as stated in SOW: 1, 2, 4, 5, 6, 7 and 8. For Specific Aim 1, we have synthesized 18 QSI-PAMAM complexes by encapsulation (Task 1), conjugation (Task 2), or both. For Specific Aim 2, we have established all the protocols to measure planktonic and biofilm growth of PA (Task 5), turnover of PAMAM in PA (Task 6), quinolone signals by HPLC-MS (Task 7), and extracellular virulence factors (Task 8). The most significant finding was the specific inhibition of PAMAM dendrimers on *Pseudomonas aeruginosa* (PA) biofilm formation and growth. This is significant because PA in the biofilm form is known to be up to 1000 fold more resistant antibiotic treatments. We systematically analyzed the effects of surface charge and particle size on PA biofilm. PAMAM dendrimers with more positive charges on the surface and bigger particle sizes exhibited more potent inhibition. Confocal fluorescent microscopy showed that PAMAM dendrimers also altered the morphology of PA biofilm. In addition, combination of PAMAM dendrimers also significantly enhanced the inhibition of PA biofilm growth by antibiotic such as amikacin.

Two new papers came out recently on the identification of PqsR antagonists (14,15). These compounds, similar to the AA analogs, block PA quinolone signaling. The target of these compounds is PqsR, which activates quinolone signal synthesis and downstream virulence factor production. Therefore, they interfere with both quinolone synthesis and

signaling, presenting some advantages over inhibitors that only block either synthesis or signaling process. In reviewing their chemical structures, they are hydrophobic compounds that may have solubility issues, hindering the efficacy *in vivo*. Therefore we will acquire the commercially available PqsR antagonists and test whether complexation with PAMAM will enhance their activities in inhibiting PA quinolone signaling.

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SUPPORTING DATA:

Table 1. Summary of QSI-PAMAM complexes synthesized in this performance period.

N°	Complex ID	QSI	Method of Complexation	QSI/PAMAM ratio					Amount (mg)
				G4-PEG	G5-NH_2	G5-Ac25	G5-Ac50	G5-Ac75	
1	B3-6	6CABA	E[a]					9	14
2	B3-7	6CABA	E		76				10.3
3	B3-8	6CABA	E	31					7
4	B3-17	6CABA	E		65				24.7
5	B3-11	6FABA	E		59				15.4
6	B3-13	SA	E		89				24.2
7	B3-14	SA	E					6	10
8	B3-15	4CABA	E		45				18.4
9	B3-18	3CABA	E		51				14.4
10	B3-24	Baicalin	C[b]				4		32.8
11	B4-5	Baicalin	C				12		31
12	B4-6	Baicalin	E, C				40[c]		50.6
13	B4-31	6FABA	E			56			20.2
14	B4-32	6CABA	E			54			22.3
15	B4-33	4CABA	E			18			18
16	B4-34	6FABA	E				40		26.9
17	B4-35	6CABA	E				64		32.6
18	B4-36	4CABA	E				20		25.5

[a] E: encapsulation; [b] C: conjugation.

[c] B4-6 complex was formed by encapsulate more baicalin molecules on the surface of B4-5. The final QSI/PAMAM ratio is the total from both encapsulation (28) and conjugation (12).

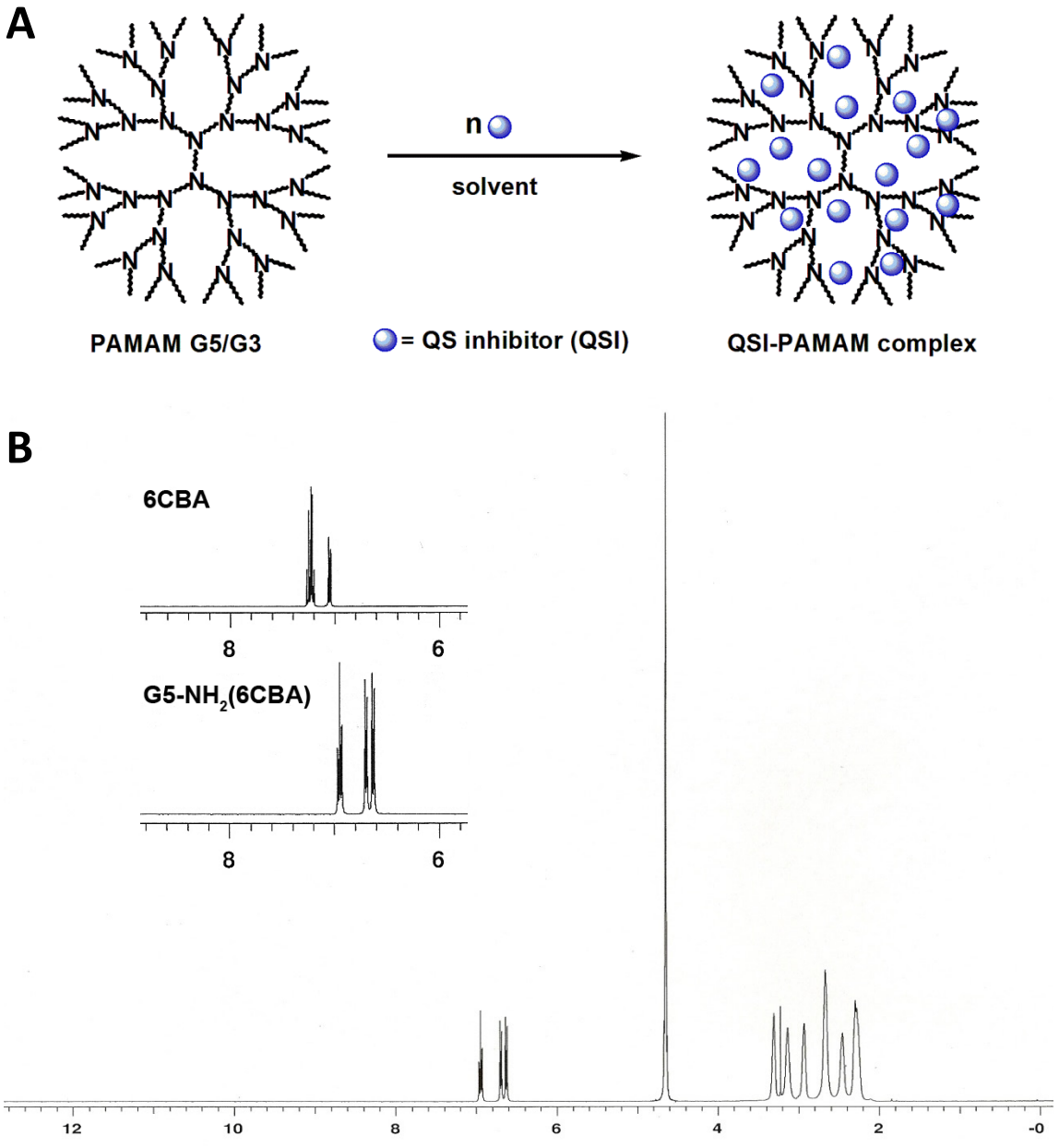

Figure 1. Encapsulation scheme (**A**) and ^{1}H NMR spectrum of G5 PAMAM encapsulated 6CABA (**B**). The inset of panel B shows the chemical shift of 6CABA after encapsulation.

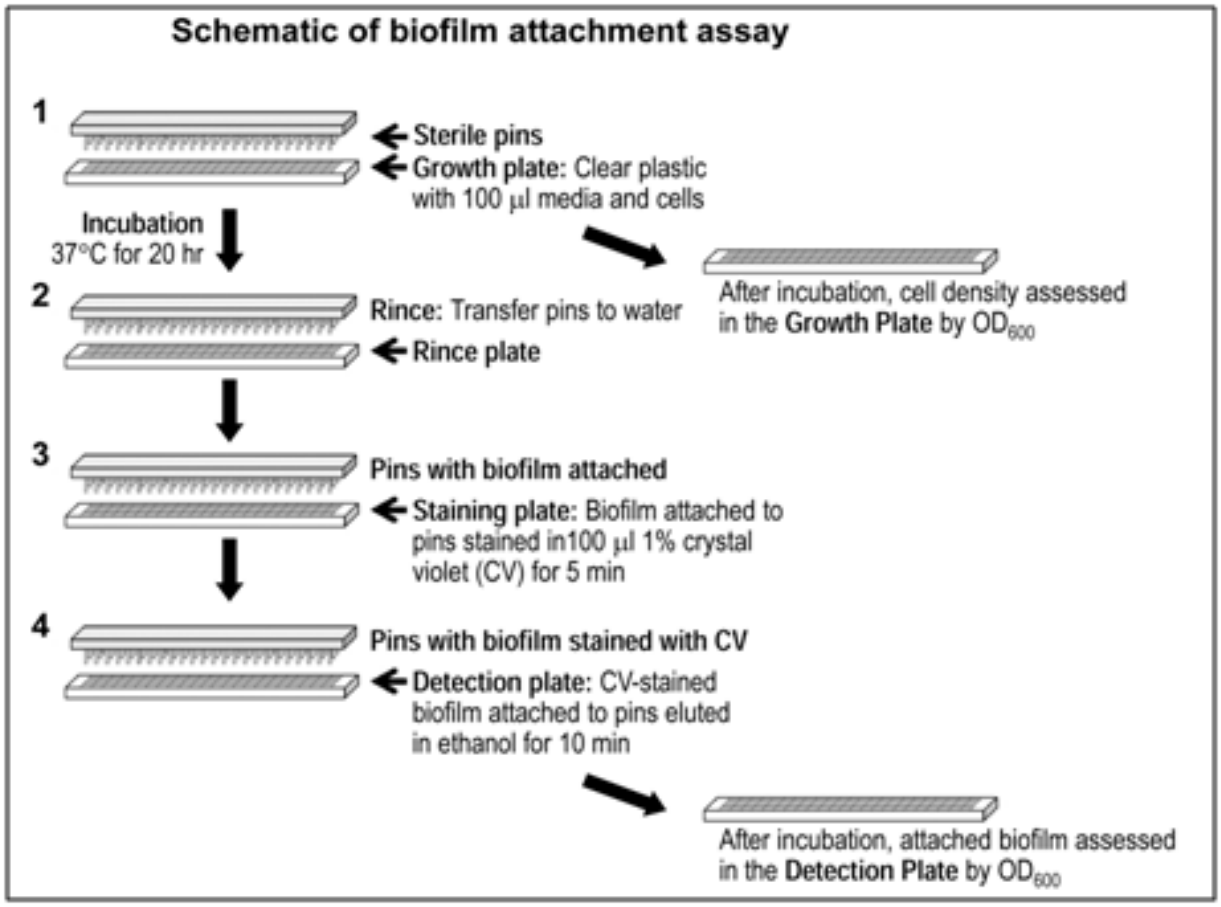

Figure 2. Workflow of pegged-lid method for biofilm formation. PAO1 is grown overnight in LB, diluted with fresh media, and inoculated in individual wells of the growth plate. Different types of dendrimers are pipetted into corresponding wells and a pegged lid is then placed over the top of growth plate. After incubation at 37°C for 20 h, the biofilms that attached on the pegged lid are stained with crystal violet and then de-stained with 100% ethanol. The optical density of both the planktonic growth within the growth plate and the biofilm growth within the detection plate is determined at 600 nm.

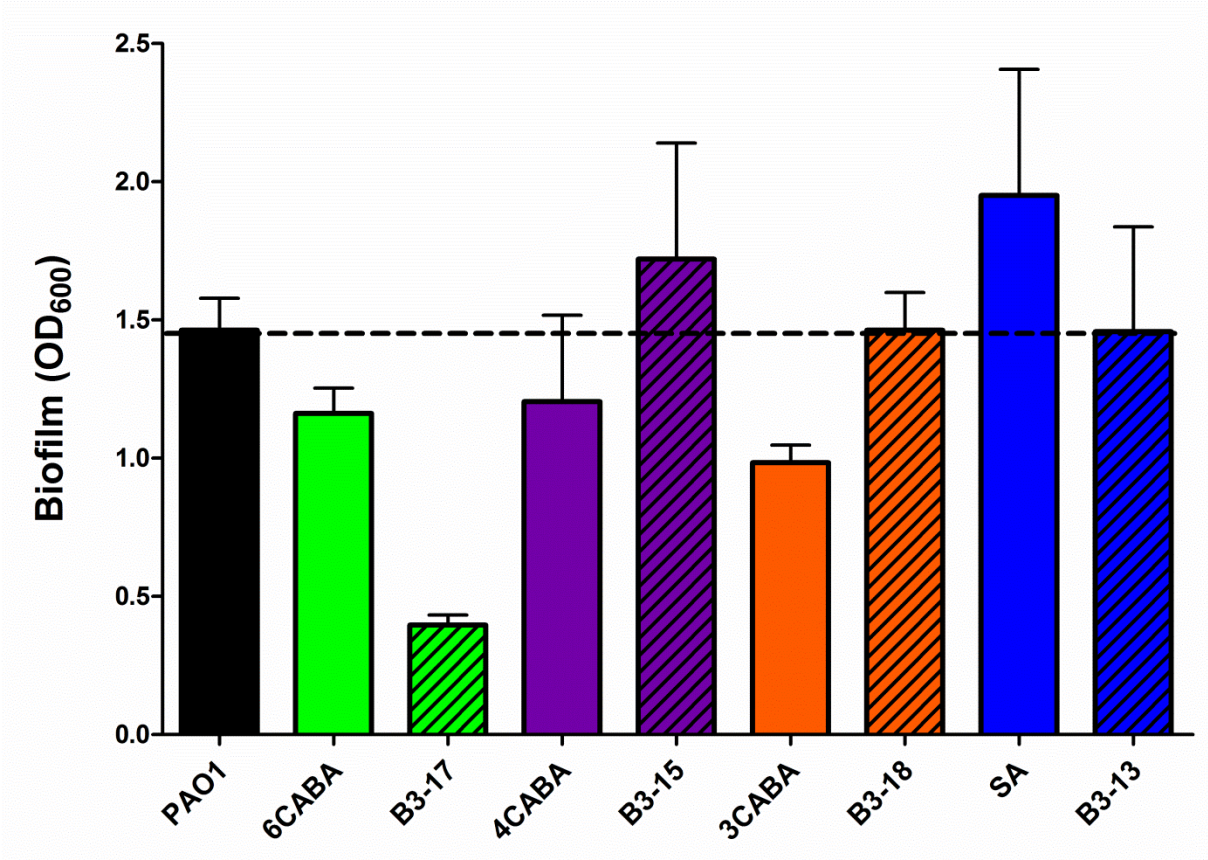

Figure 3. Comparison of QSI and QSI-PAMAM complex on PA biofilm growth. Encapsulation of 6CABA (green bars) enhanced the inhibition on biofilm.

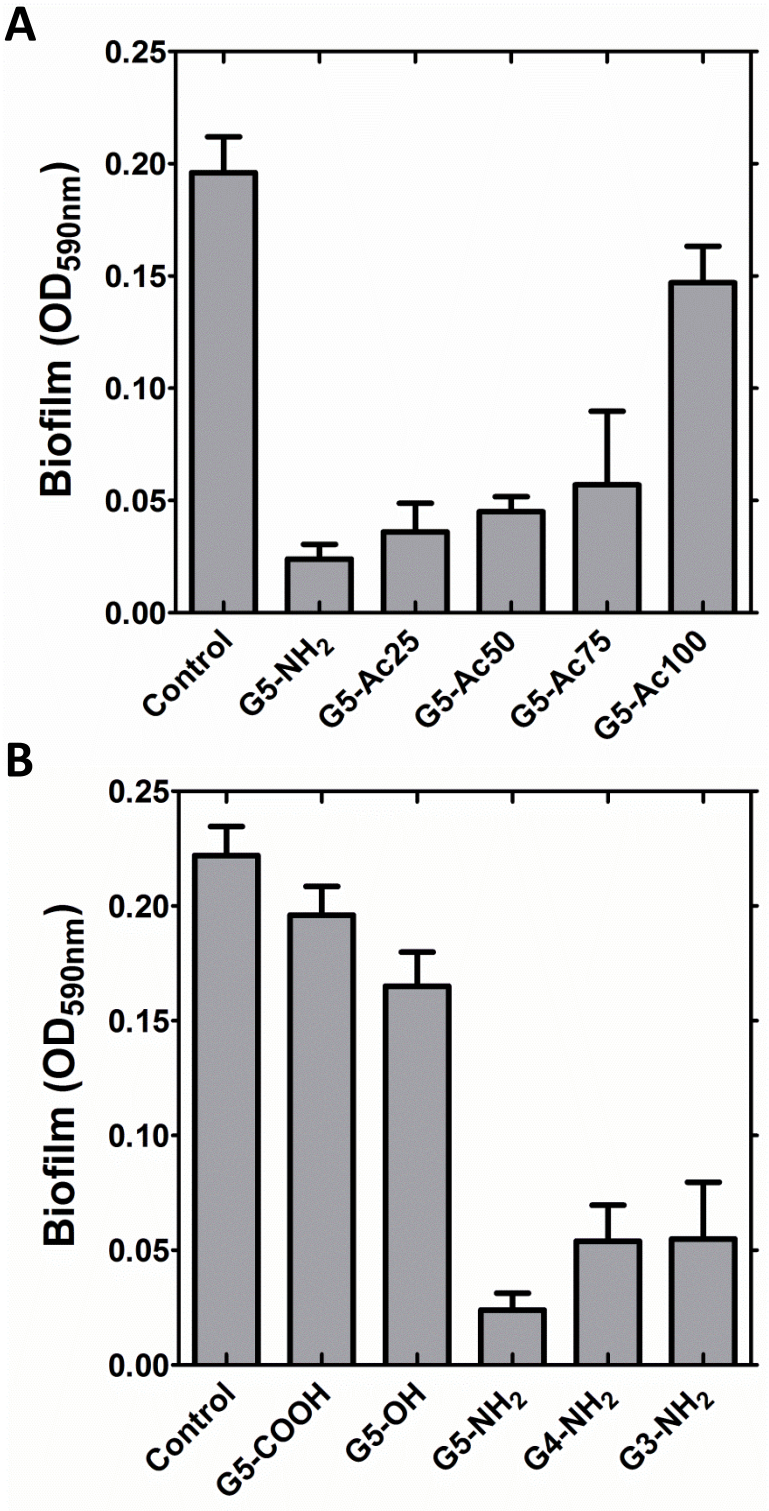

Figure 4. Effects of various PAMAM dendrimers on PA biofilm growth. (**A**) Comparison of G5 dendrimers with different surface positive charges. (**B**) Comparison of PAMAM with different surface charge and particle size. All dendrimers were tested at 20 μM.

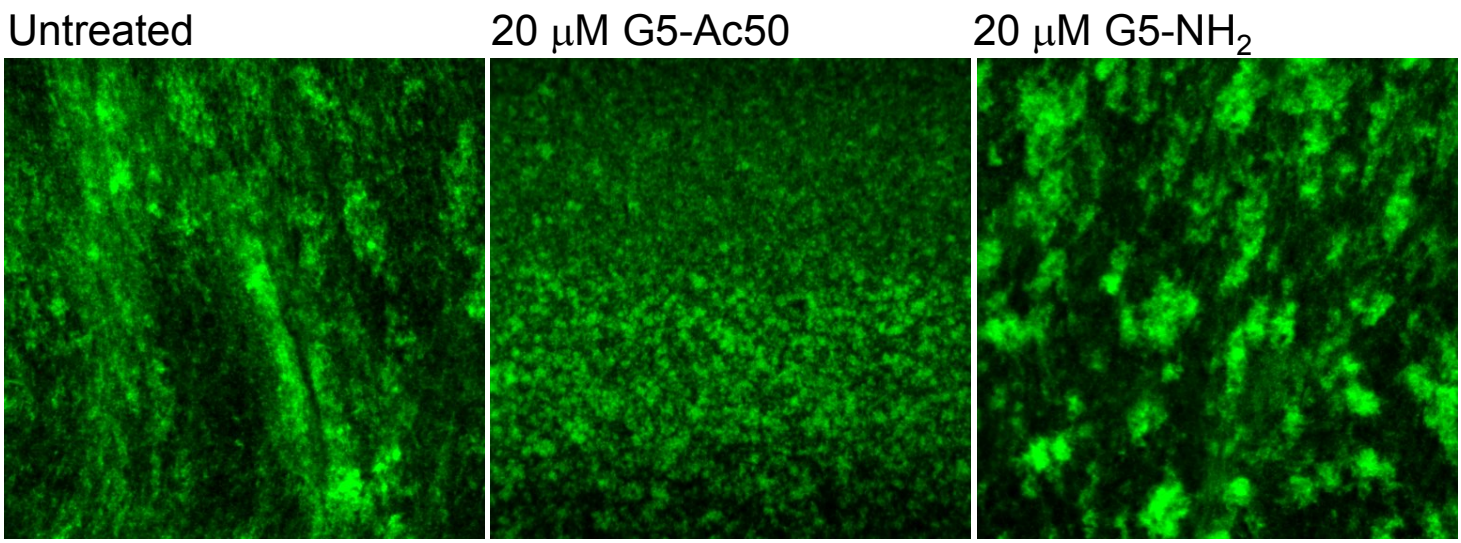

Figure 5. PAMAM dendrimers altered the morphology of PA biofilm.Strain PAO1 carrying GFP-expressing plasmid (pSMC2) was used. The morphology of the biofilm was monitored by confocal fluorescent microscopy.

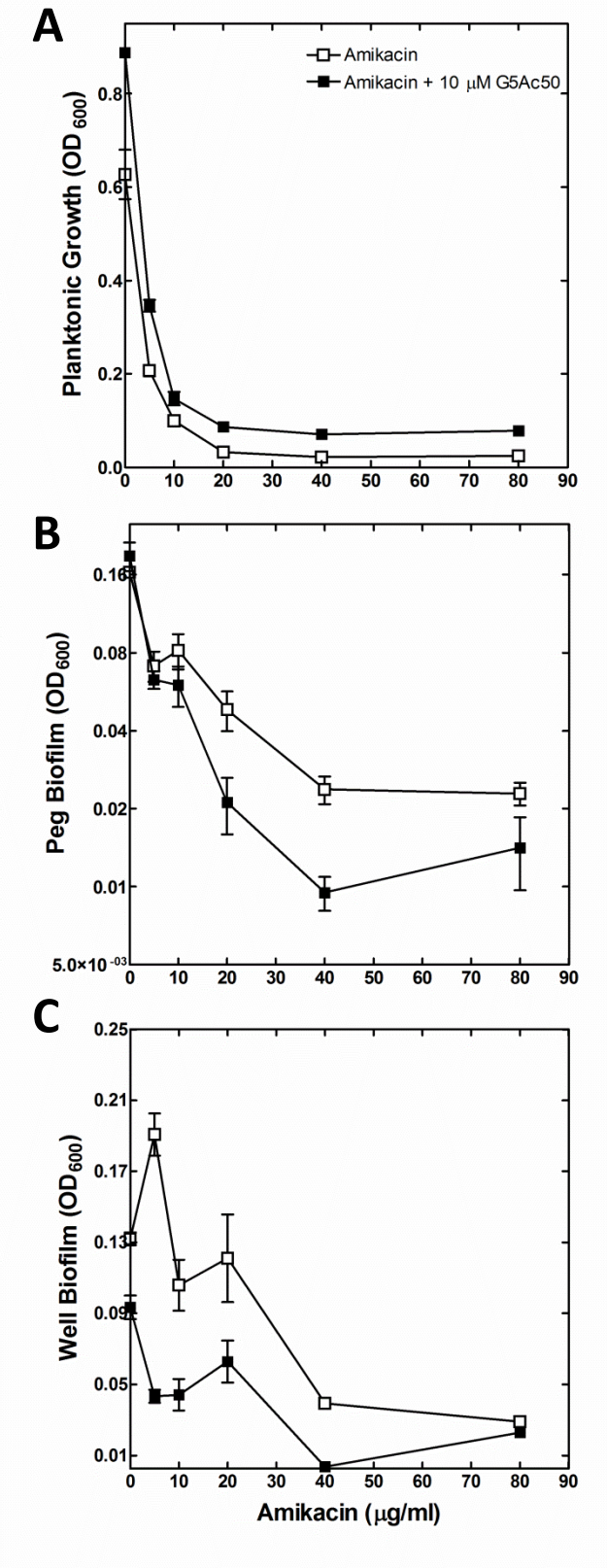

Figure 6. G5-Ac50 significantly improved the inhibitory effect of amikacin on PA biofilm formation (**B**, **C**). No synergistic effect was observed on amikacin inhibition on PA planktonic growth (**A**).

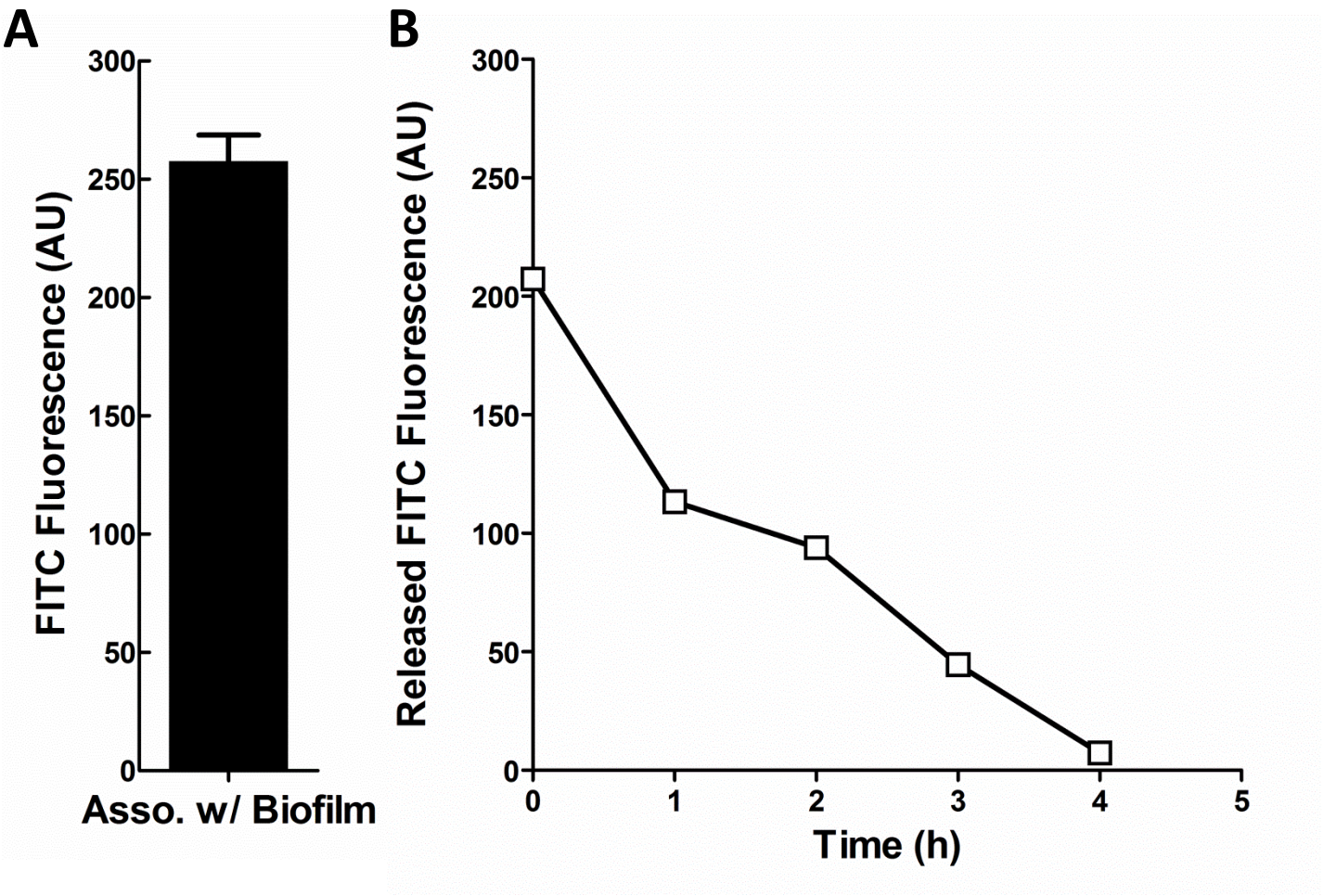

Figure 7. Uptake of G5 PAMAM dendrimer by PA. (**A**) About 8% of total FITC-labeled G5 dendrimer was associated with PA biofilm after 2 hr incubation. (**B**) FITC-G5 was released from PA biofilm.

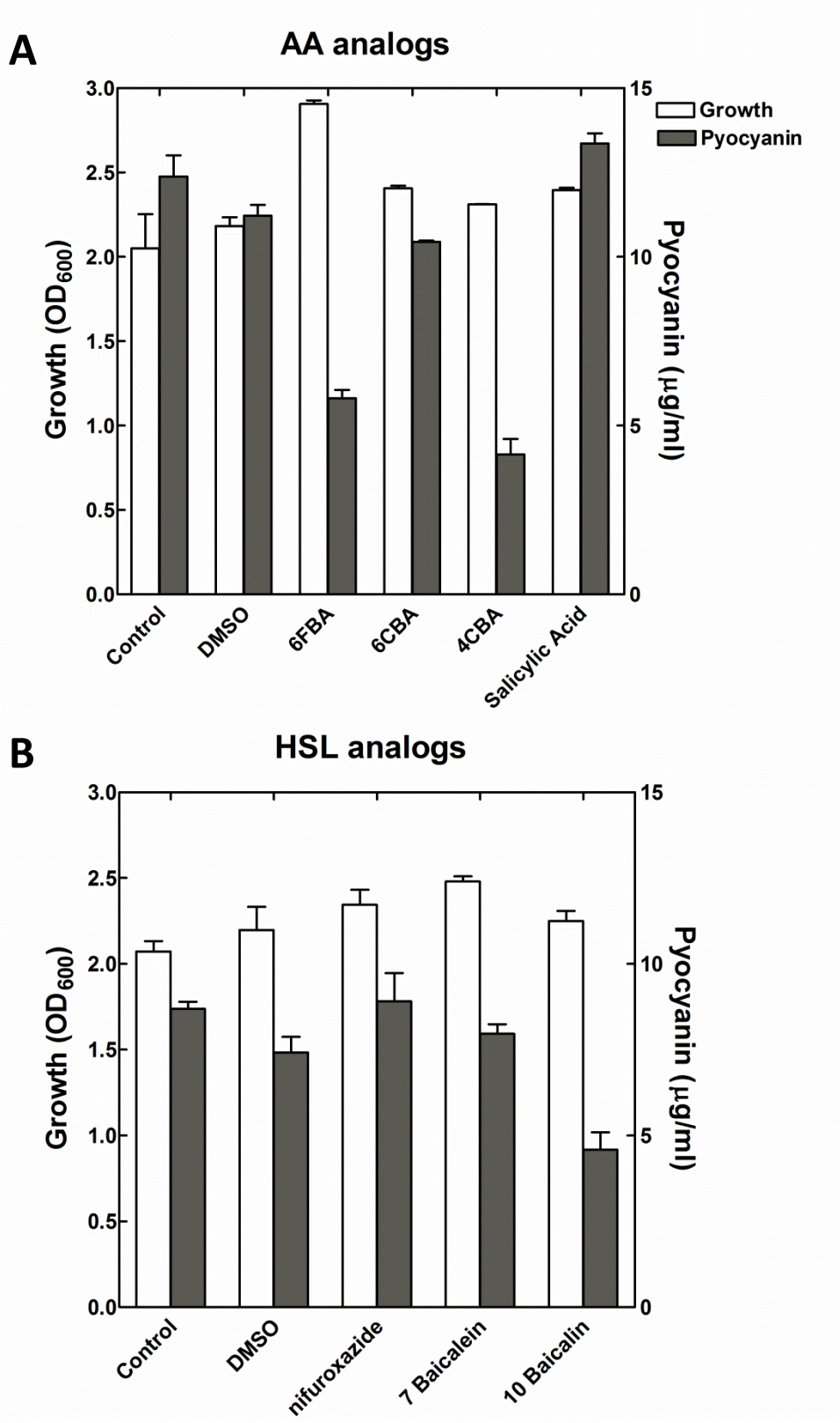

Figure 8. Effects of anthranilic acid (AA) analogs (**A**) and homoserine lactone (HSL) analogs (**B**) on PA growth (open bars) and pyocyanin production (grey bars).